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First record of genus *Cryptonanus* (Didelphimorphia) in the state of Rio de Janeiro, Brazil

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Abstract: Here we report the first record of genus *Cryptonanus* in the state of Rio de Janeiro, Brazil. One specimen (MZUSP35409) was captured in an Atlantic Forest fragment and was identified by morphological characters and molecular analysis using cytochrome-b (*MT-CYB*) and von Willebrand Factor (*VWF*). Phylogenetic reconstructions based on *VWF* sequences positioned our specimen into the genus *Cryptonanus*, and the shortest genetic distance of *MT-CYB* was estimated between our specimen and an undescribed specimen from Piauí. Our record increases the genus' geographic range approximately 350 km from Cotia and Ibiúna municipalities, São Paulo. The genus *Cryptonanus* remains taxonomically poorly understood.

Key words: Atlantic Forest; Didelphidae; molecular analysis; *MT-CYB*; *VWF*

The recently described genus *Cryptonanus* Voss, Lunde & Jansa, 2005 has been included in the monophyletic group of small didelphid marsupials of the Tribe Thylamyini (Voss and Jansa 2009). This genus comprises species formerly assigned to *Gracilinanus* (Voss et al. 2005; Lóss et al. 2011). An accurate examination of morphological, karyotypic, and molecular characters allowed the description of this new genus (Voss et al. 2005).

Cryptonanus species are characterized by their small size, dark circumocular masks, long prehensile tails, and lack of pouch (Voss et al. 2005). *Cryptonanus* differs from *Gracilinanus* mainly by lack of a rostral process in premaxillae, a secondary foramen ovale and maxillary fenestrae, second upper premolar shorter than the third one, and the presence of accessory cusps in upper canines (Voss et al. 2005; Voss and Jansa 2009; Garcia et al. 2010).

Currently, five species of *Cryptonanus* have been described: *C. agricolai* (Moojen, 1943), *C. chacoensis* (Tate, 1931), *C. guahybae* (Tate, 1931), *C. ignitus* (Díaz, Flores, and Barquez, 2002), and *C. unduaviensis* (Tate, 1931) (Voss et al. 2005). *Cryptonanus* is mainly distributed in unforested tropical and subtropical biomes south of the Amazonas River and east of the Andes, in the Brazilian biomes of Caatinga, Cerrado, Chaco, and Pampas (Voss and Jansa 2009), with recent records in the Atlantic Forest (Umetsu and Pardini 2007; Quintela et al. 2011, Dias et al. 2015). *Cryptonanus agricolai* occurs in Caatinga and Cerrado of east-central Brazil and *C. chacoensis* in Mato Grosso and Mato Grosso do Sul states in Brazil, northern Argentina and Paraguay, and probably Uruguay (Gardner 2007; D'Elía and Martínez 2006; Bezerra et al. 2009). On the other hand, the distribution of the other species is more restricted. *Cryptonanus guahybae* occurs in Rio Grande do Sul, Santa Catarina and Paraná states, Brazil, *C. unduaviensis* in eastern Bolivia, and the presumably extinct *C. ignitus*, in its type locality in Jujuy, Argentina (Díaz et al. 2002; Gardner 2007; Voss et al. 2005; Dias et al. 2015).

To date, at least two records of *Cryptonanus* were reported in southeastern Brazil (São Paulo State). A non-described species was recorded in eucalyptus plantations in Caucaia do Alto, located in Cotia and Ibiúna municipalities (Umetsu and Pardini 2007). In Angatuba municipality, specimens of *C. agricolai* were recorded in three habitat types (native vegetation, abandoned pasture, and eucalyptus plantation) in a farm located in the transitional zone between the semi-deciduous Atlantic forest and Cerrado (Martin et al.

2012). Recent taxonomic studies showed that specimens previously identified as *Gracilinanus* may actually belong to *Cryptonanus*, and that three likely species of this genus may occur in the state of São Paulo (Vivo et al. 2011). Here we report the first record of *Cryptonanus* in the state of Rio de Janeiro (Southeastern Brazil).

The study was carried out in Serra das Araras, Piraí municipality, state of Rio de Janeiro, Brazil. Five sites of ombrophilous dense forest located in the center of Tinguá-Bocaina Biodiversity Corridor were sampled in February 2013. Non-volant small mammals were sampled with livetraps (Sherman®) and pitfall traps (IBAMA/MMA process no. 02001.007478/2008-41, authorization no. 196/2012).

At each sampling site, one 90 m transect was established, with 10 trap stations. Each station had one livetrap placed on the ground and one placed in the understory between 1.5 and 2.5 m above ground, with a total sampling effort of 500 traps-night. Pitfall traps consisted of 60 L plastic buckets arranged in transects and placed 10 m apart. Buckets were connected by a plastic-sheet drift fence 0.5 m high, buried 0.1 m below and extended perpendicularly to the ground in order to induce the capture of wandering individuals. Each of the five sampling site had eleven buckets, and the total sampling effort was 275 bucket-nights.

All specimens trapped were identified at the species level whenever possible, weighed using spring scales, sexed, measured (heady-body and tail lengths), and marked with a numbered ear-tag on first capture (Ear Tags, National Band & Tag Co., Newport, Kentucky, USA). Unidentified specimens were collected, euthanized, prepared, and deposited in Museu de Zoologia da Universidade de São Paulo (MZUSP). Liver samples were collected and preserved in 100% ethanol and deposited in Laboratório de Vertebrados da Universidade Federal do Rio de Janeiro. For marsupials, teeth eruption and functionality pattern permitted accurate age estimation (Macedo et al. 2006). Following Macedo et al. (2006), specimens were classified as young (dentition dPxMx), subadults (P3M3/4), or adults (P3M4/4), where d= deciduous tooth, P= premolar, and M= molar.

DNA was isolated from the liver of one specimen with the phenol-chlorophorm protocol (Sambrook et al. 1989). Cytochrome b (*MT-CYB*) DNA was analyzed for inferring relationships between con-generic species and a partial sequence data of exon 28 of the von Willebrand Factor (*e28-VWF*) for inferring relationships between different genera. Gene nomenclature followed HUGO Gene Nomenclature Committee (http://www.genenames.org).

MT-CYB DNA was PCR amplified with primers L14724 (Irwin et al. 1991) and citb REV (Casado et al. 2010), with a pre-denaturation step at 94°C for 2 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1 min 30 s, and final extension of 72°C for 5 min. *VWF* was PCR amplified with e28-vWF F104 and e28-vWF R1141 (Voss and Jansa 2009), with a pre-denaturation step at 94°C for 2 min; 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 30 s, extension at 72°C for 1 min 30 s, and final extension of 72°C for 10 min. PCR products were purified with GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Brazil). Sequence reactions were performed with PCR primers, plus CB-in1 and CB-in2 (Cassens et al. 2000), MVZ16 (Silva and Patton 1993) and MEU1 (Gonçalves et al. 2005) for *MT-CYB*, e28-vWF F120, e28-vWF R655 and e28-vWF R743 for *VWF*, labeled with XL and BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and loaded to an ABI Prism™ 3130 platform.

Sequences were edited, assembled with Chromas (McCarthy 1998) and Bioedit (Hall 1999), aligned with MEGA v. 6 (Tamura et al. 2011) and deposited in GenBank (KT334295 and KT334294). Phylogenetic analyses based on *MT-CYB* included GenBank data from other *Cryptonanus* species (*C. agricolai*, *C. chacoensis, C. guahybae* and *C. unduaviensis,* and one *Cryptonanus* sp.), with *Gracilinanus microtarsus*, *Marmosa murina*, *Marmosops paulensis* and *Thylamys macrurus* as outgroups (Table 1). Analyses of *VWF* data were carried out following

Table 1. Mitochondrial Cytochrome b sequences utilized in this study, with Museum or field number, GenBank accession number, locality and reference of published study. BR = Brazil; BOL = Bolivia; PAR = Paraguay.

Species	Voucher or catalog number	GenBank accession number	Locality	Reference
Cryptonanus sp. 1	MZUSP 35409	KT334295	BR: RJ, Piraí, Serra das Araras	This study
Cryptonanus sp. 2	UUPI167	HQ622148	BR: PI, Uruçui, Uruçui-Una Ecological Station	Lóss et al. (2011)
Cryptonanus agricolai	LBCE7486	KF313984	BR: GO, Serranópolis	Faria et al. (2013)
Cryptonanus chacoensis	GD 521	GQ911596	PY, no details	Teta et al. (2009)
Cryptonanus guahybae	UFSC4854	KM403638	BR: SC, Palhoça, Baixada do Maciambu	Dias et al. (2015)
Cryptonanus guahybae	MCNU2808	KM403639	BR: RS, Três Passos	Dias et al. (2015)
Cryptonanus guahybae	MCNU1725	KM403640	BR: RS, Esmeralda, Aracuri Ecological Station	Dias et al. (2015)
Cryptonanus unduaviensis	NK14234	HM583366	BO: Pando, Independencia	Giarla et al. (2010)
Gracilinanus microtarsus	LBCE6655	KF313982	BR: RJ, Teresópolis	Faria et al. (2013)
Marmosa murina	USNM 549292	HM106395	BR: PA, E bank Rio Xingu	Gutiérrez et al. (2010)
Marmosops paulensis	LBCE7437	KF313983	BR: RJ, Teresópolis	Faria et al. (2013)
Thylamys macrurus	NK27536	HM583383	PY: Concepción	Giarla et al. (2010)

Voss and Jansa (2009), comparing different marsupial genera to establish the inter-generic relationships and the position of *Cryptonanus* in the topology. This included our GenBank data from *C. agricolai* (KF314038), *C. chacoensis* (FJ159331) and *C. unduaviensis* (FJ159332), and additional 47 sequences from other marsupials (AY243403, AY243405, AY243407, AY243411, AY243413, FJ159328-70, KF314037).

Genetic distance estimates were carried out with complete deletion using Kimura's two parameters, with MEGA v. 6. For phylogenetic reconstructions, the best fit model of evolution was inferred with ModelGenerator v. 0.85 (Keane et al. 2006), according to Akaike Information Criterion 2 (AIC; Burnham and Anderson 2002; Keane et al. 2006). For *MT-CYB*, the best fit model was the generalized time reversible model, with proportion of invariable sites estimated and gamma distributed rate (GTR+I+G). For *VWF*, the best fit model was the transversion model with equal base frequencies and gamma distributed rate (TVMef+G).

Phylogenetic reconstructions were performed with PHYML 3.0 (Guindon et al. 2010) for maximum likelihood (ML) with bootstrap estimates based on 1,000 replicates. Branch support was also calculated using the approximate likelihood ratio test (aLRT) with SH-like interpretation. Bayesian analyses (BA) were carried out with MrBayes 3.2.1 (Ronquist and Huelsenbeck 2003). Posterior probabilities values were estimated sampling every 100th generation over a total of 1,000,000 with a burn-in of 25% generations (2500 trees). Effective sample size (ESS) and convergence diagnostic values were considered when above 100 and 1, respectively. Topologies were generated and edited with FigTree v1.4.0 (Rambaut 2012). DNAsp 5 was used for haplotype estimates and nucleotide diversity (Librado and Rozas 2009). Following Voss and Jansa (2009) we assumed the monophyly of tribe Thylamyini considering *Marmosa murina* and *Marmosops paulensis* as the most basal offshoots of the topology.

Figure 1. Specimen of *Cryptonanus* (MZUSP35409) captured in a pitfall trap in Serra das Araras, municipality of Piraí, RJ, Brazil. Photo: Ana C. Delciellos.

One specimen of *Cryptonanus* (MZUSP 35409; Figure 1) was captured in Serra das Araras (22°39′10″ S, 043°50′26″ W, datum SAD69, altitude ca. 500 m), municipality of Piraí, Rio de Janeiro state, Brazil, on 4 February 2013 (Figure 2). This specimen was a young male (dentition dP3M2/3 following Macedo et al. 2006) captured in a pitfall trap, in a disturbed forest fragment of secondary vegetation, with an open understory and predominance of small diameter trees (< 15 cm).

This specimen was morphologically identified to belong to *Cryptonanus* by the following anatomical features: presence of prehensile tail, tail longer than head-body length, lack of maxillary fenestrae, rostral process in premaxillae and secondary foramen ovale (Table 2; Figures 1 and 3). Apparently, there were no accessory cusps in the upper canines.

Sequence data accounted for 1,146 bp of *MT-CYB* and 865 bp of *VWF* but phylogenetic reconstructions were limited to a 713 bp and 836 bp of each gene, respectively, in view of the dearth of data available in GenBank. Genetic distance estimated between *MT-CYB* of *Cryptonanus* species ranged from 0.008 to 0.129 (Table 3) and between the two *Cryptonanus* sp. was

Table 2. Comparison of diagnostic morphological and cranial features between described Brazilian *Cryptonanus* species and the specimen of *Cryptonanus* captured in this study (MZUSP 35409).

	C. agricolai	***C. chacoensis***	***C. guahybae***	***Cryptonanus* sp. 1 (MZUSP 35409)**
Head-Body length (mm)	74–95	82–114	72–94	60*
Tail length (mm)	90–114	107–136	106–118	84*
Rate TA/HB	1.03–1.4	1.12–1.42	1.21–1.44	1.4
Length of body hair	Short	Short	Long	Long(?), dorsal hair about 7.5 mm in length
Dorsal pelage coloration	Brown-gray	Brown-gray	Brown-gray	Brown-gray
Ventral pelage coloration	Self-whitish or cream inconspicuously gray-based	Self-whitish or with gray-based hairs	Cream gray-based	Cream gray-based
Molar toothrow (mm)	4.9–5.4	4.9–5.5	5.2–5.3	5.0*
Weight (g)	18.0	15.5	?	6.0*
References	Voss et al. (2005); Voss and Jansa (2009); Rossi et al. (2012)	Voss et al. (2005); Voss and Jansa (2009); Rossi et al. (2012)	Voss et al. (2005); Voss and Jansa (2009); Rossi et al. (2012)	This study

*The specimen was classified as young, following Macedo et al. (2006).

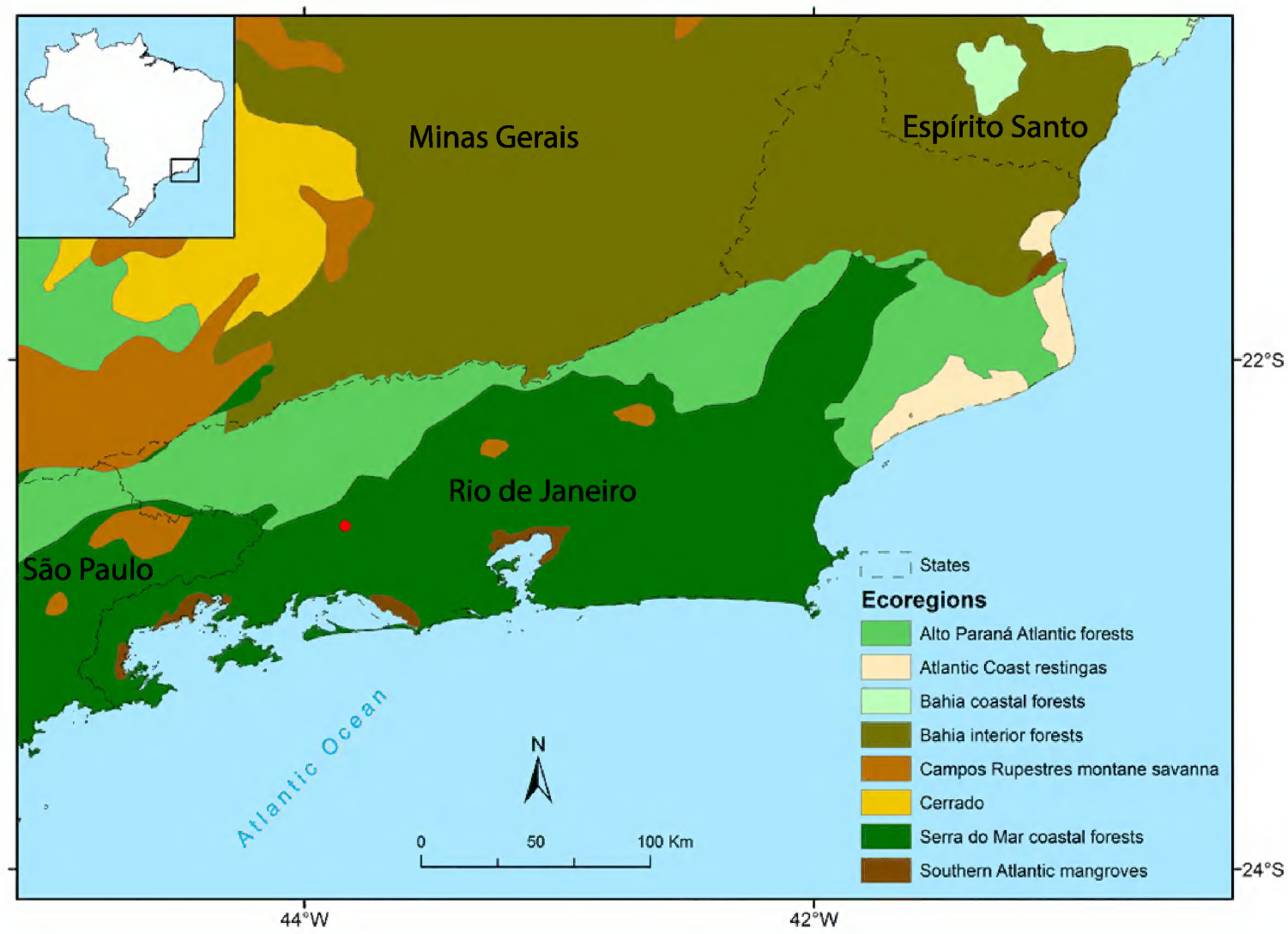

Figure 2. Record of a *Cryptonanus* specimen (red circle; MZUSP35409) in the state of Rio de Janeiro, Brazil.

0.032. Distance between *C. chacoensis* and *C. agricolai* was 0.008 while distances between the three specimens of *C. guahybae* ranged from 0.002 to 0.003 (Table 3).

Phylogenetic reconstructions based on *MT-CYB* showed two main lineages splitting from a strongly supported node; the one leading to *C. unduaviensis* and the second one to the clade (((*C. chacoensis*, *C. agricolai*) (*Cryptonanus* sp. 1, *Cryptonanus* sp. 2)) *C. guahybae*) (Figure 4). Effective sample size (ESS) values were above 100, indicating that the parameter was not undersampled. The convergence diagnostic (PSRF, potential scale reduction factor) showed values above 1. Reconstructions based on *VWF* positioned our specimen in *Cryptonanus*.

The specimen herein identified is the first record of the genus *Cryptonanus* in the state of Rio de Janeiro, in addition to others in southeastern Brazil (Umetsu and Pardini 2007; Martin et al. 2012), a region previously considered outside the range of this genus. Species of *Cryptonanus* are frequently described as inhabitants of open areas (Voss and Jansa 2009), but the establishment of large plantations with open understory, and the accelerated loss of habitat and fragmentation have probably contributed to the appearance of this genus in the southeastern region of Brazil (Umetsu and Pardini 2007). Our record increases the geographic range of this genus by approximately 350 km from the nearest previous records in Cotia and Ibiúna municipalities, state of São Paulo (Umetsu and Pardini 2007).

Cryptonanus sp. from Serra das Araras showed diagnostic cranial characters of this genus like lack of maxillary fenestrae, secondary foramen ovale and rostral process in premaxillae (Voss et al. 2005; Voss and Jansa 2009; Garcia et al. 2010). Apparently, accessory cusps in the upper canines were absent, as is the case of other specimens of *Cryptonanus* (Voss et al. 2005). The precise identification of this specimen still requires further analysis. A preliminary comparison of other anatomical features between this young and not fully developed specimen and the three Brazilian *Cryptonanus* species has been inconclusive.

Molecular analyses corroborated the phylogenetic position of this specimen in *Cryptonanus*. Measures of genetic distances are an important tool in the assessment

Table 3. Genetic distance estimates carried out with complete deletion using Kimura's two parameters among Cytochrome b sequences of *Cryptonanus* species.

	Taxa	1	2	3	4	5	6	7
1	*Cryptonanus* sp. 1 (MZUSP35409)							
2	*Cryptonanus* sp. 2 (HQ622148)	0.032						
3	*C. chacoensis*	0.053	0.065					
4	*C. agricolai*	0.055	0.067	0.008				
5	*C. unduaviensis*	0.109	0.119	0.104	0.104			
6	*C. guahybae* (KM403639)	0.121	0.127	0.104	0.104	0.123		
7	*C. guahybae* (KM403640)	0.123	0.129	0.106	0.106	0.124	0.003	
8	*C. guahybae* (KM403638)	0.123	0.129	0.106	0.106	0.125	0.002	0.002

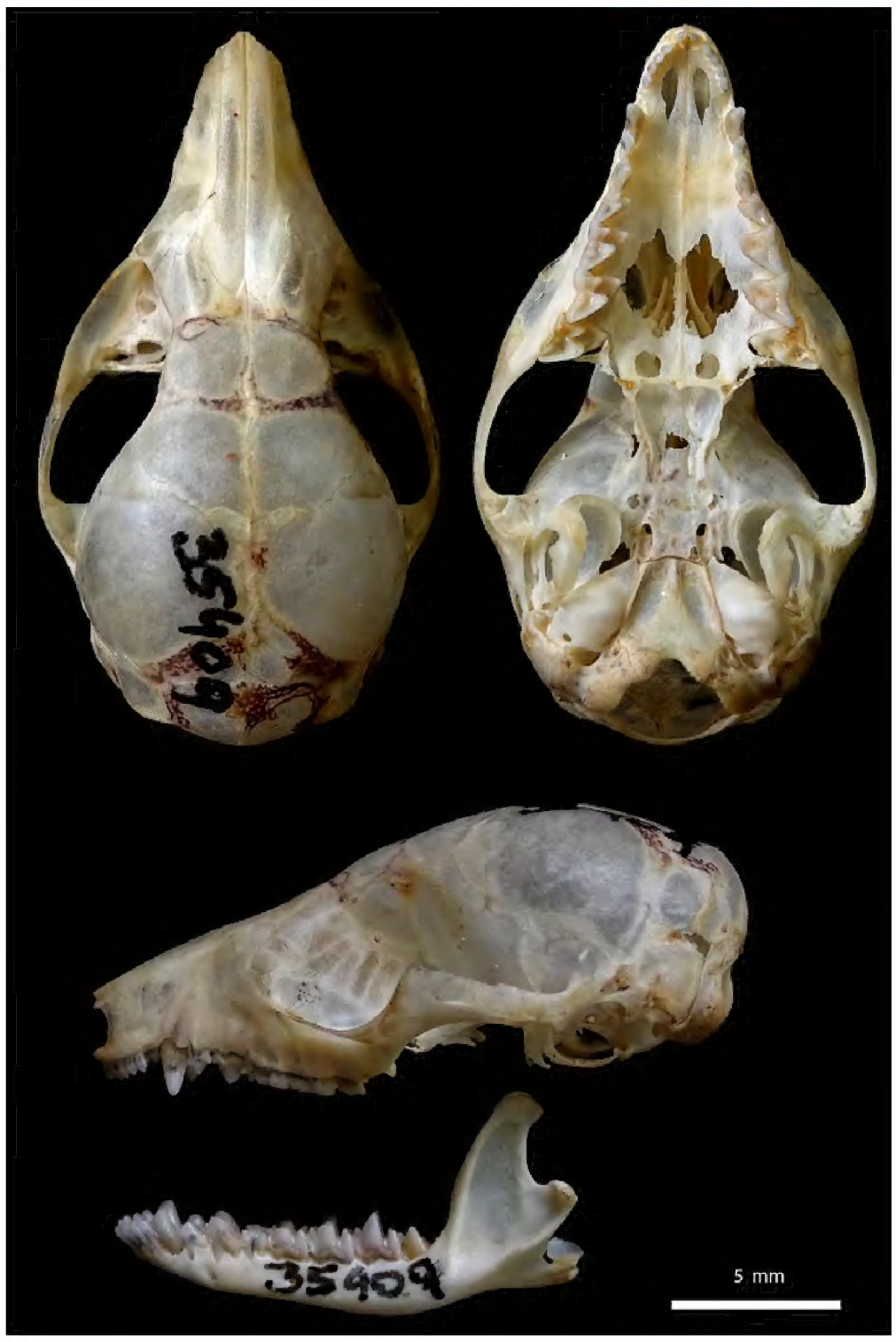

Figure 3. Dorsal, ventral, and lateral views of the skull, and lateral view of the mandible of the *Cryptonanus* specimen (MZUSP35409) from Serra das Araras, municipally of Piraí, state of Rio de Janeiro, Brazil.

of genetic diversity. For the marsupials analyzed here, the genetic distance of *MT-CYB* ranged from 0.008 to 0.129 between species. The genetic distance between the two *Cryptonanus* sp. was estimated in 0.032 while the distance of 0.008 between the recognized species *C. chacoensis* and *C. agricolai* was the shortest one.

In the phylogenetic topology, our specimen from Rio de Janeiro state grouped with the *Cryptonanus* sp. captured in Uruçui-Una Ecological Station, municipality of Uruçui, state of Piauí, Brazil, 1,600 km from the municipality of Piraí, state of Rio de Janeiro. The nearest *Cryptonanus* records corresponded to specimens captured in São Paulo (Umetsu and Pardini 2007: ca. 350 km; Martin et al. 2012: ca. 470 km) and Paraná (Dias et al. 2015: ca. 745 km) states. For the specimens from São Paulo there are no molecular data available in the literature or in GenBank. The specimens of Angatuba municipality were identified as *C. agricolai* (Martin et al. 2012), and the recent record in Paraná state was attributed to *C. guahybae* (Dias et al. 2015). *Cryptonanus guahybae* specimens from southern Brazil grouped in a separate clade with support of 100% in the molecular analyses, ruling out any possibility of being identical with the specimens herein studied. Moreover, this specimen has been found to be different from *C. unduaviensis, C. guahybae*, and the poorly understood taxonomic group that includes *C. chacoensis* and *C. agricolai.*

The genus *Cryptonanus* is still poorly understood, requiring new studies to describe the new species. In view that this genus is presently distributed in southeastern Brazil, specimens previously identified as *Gracilinanus* deposited in the zoological collections should be reanalyzed.

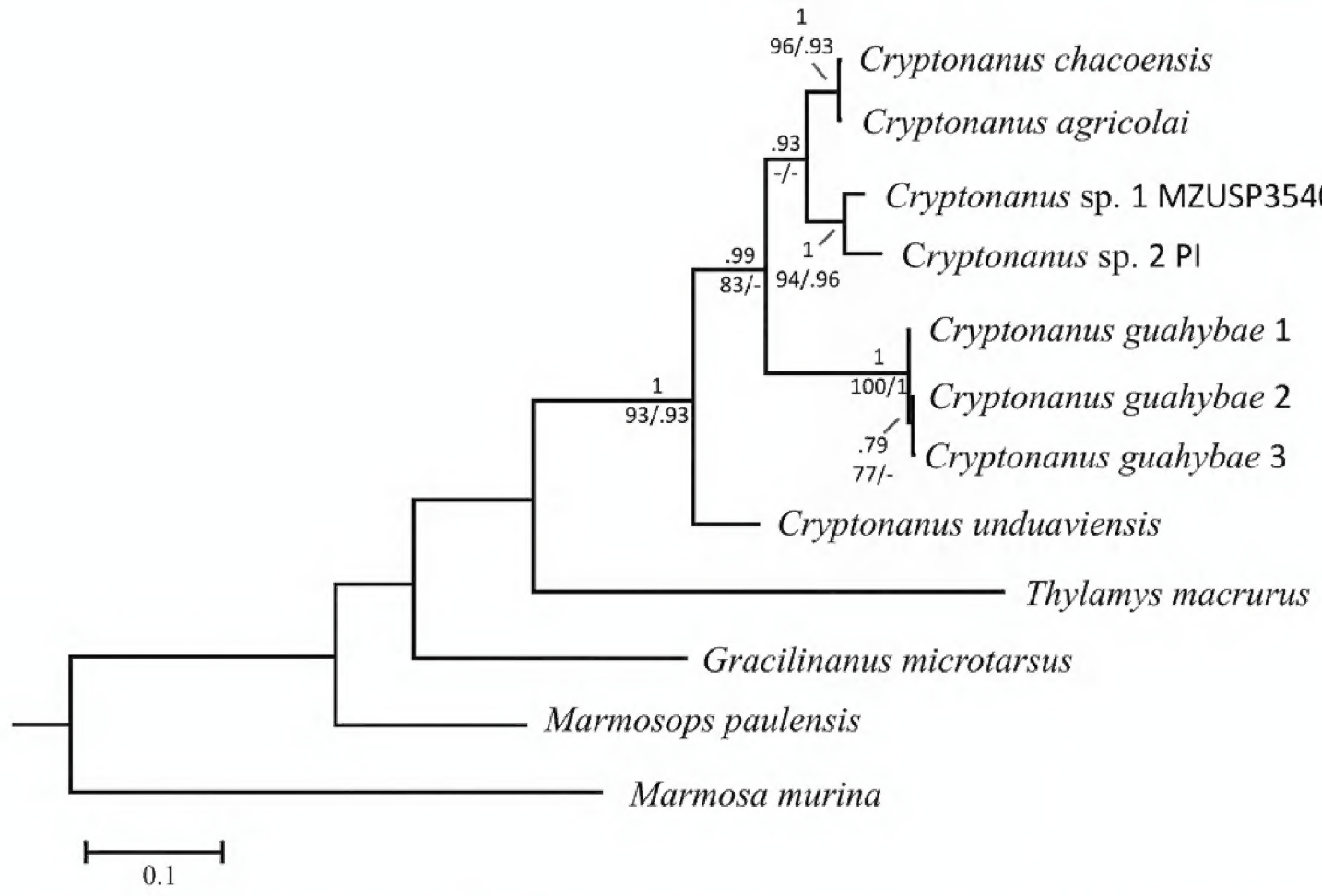

Figure 4. Phylogenetic reconstruction of mitochondrial Cytochrome b sequences carried out with GTR+I+G model, and Bayesian analysis (BA), maximum likelihood with bootstrap based on 1,000,000 replicates and approximate likelihood ratio test (aLRT). Values above branches are posterior probabilities, and below are values of bootstrap/aLRT. Low value support (> 0.8 0 for BA, > 0.9 for bootstrap and >0.75 for aLRT) are not shown. RJ = Rio de Janeiro state, Brazil; PI = Piauí state, Brazil; (1) KM403639; (2) KM403640; (3) KM403638.

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